Lignin polymerization: how do plants manage the chemistry so well?

AUTHOR(S):

Tobimatsu, Yuki; Schuetz, Mathias

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Lignin polymerization: how do plants manage the chemistry so well?

Yuki Tobimatsu[1] and Mathias Schuetz[2]

The final step of lignin biosynthesis is the polymerization of monolignols in apoplastic cell wall domains. In this process, monolignols secreted by lignifying cells, or occasionally neighboring non-lignifying and/or other lignifying cells, are activated by cell-wall-localized oxidation systems, such as laccase/O_2 and/or peroxidase/H_2O_2, for combinatorial radical coupling to make the final lignin polymers. Plants can precisely control when, where, and which types of lignin polymers are assembled at tissue and cellular levels, but do not control the polymers' exact chemical structures *per se*. Recent studies have begun to identify specific laccase and peroxidase proteins responsible for lignin polymerization in specific cell types and during different developmental stages. The coordination of polymerization machinery localization and monolignol supply is likely critical for the spatio-temporal patterning of lignin polymerization. Further advancement in this research area will continue to increase our capacity to manipulate lignin content/structure in biomass to meet our own biotechnological purposes.

Addresses
[1] Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan
[2] Department of Botany, University of British Columbia, Vancouver, BC, Canada

Corresponding authors:
Tobimatsu, Yuki (ytobimatsu@rish.kyoto-u.ac.jp),
Schuetz, Mathias (mschuetz@botany.ubc.ca)

Current Opinion in Biotechnology 2019, **56**:75–81

This review comes from a themed issue on **Plant biotechnology**

Edited by **John Ralph** and **Wout Boerjan**

https://doi.org/10.1016/j.copbio.2018.10.001

Introduction

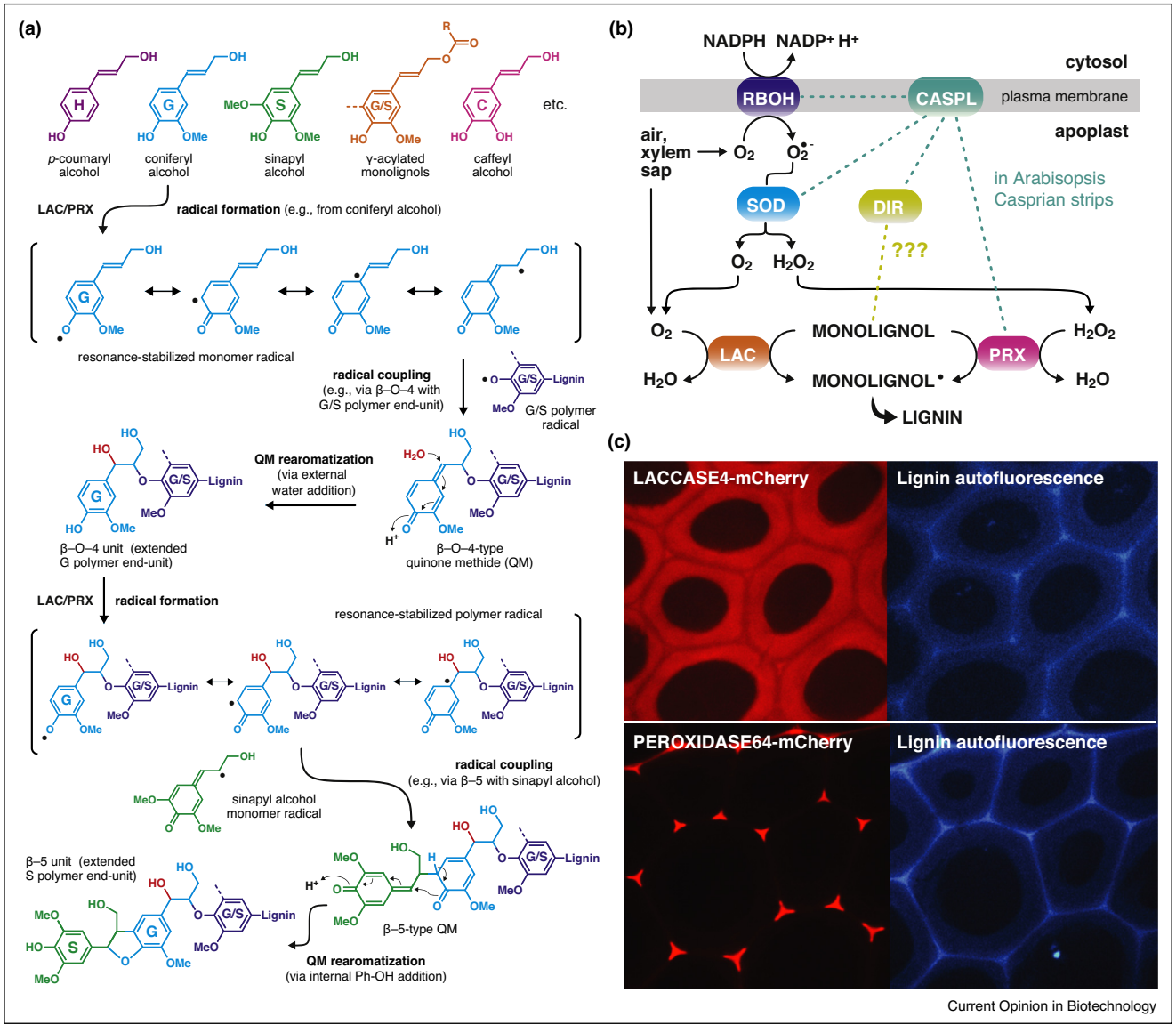
Lignin is an aromatic biopolymer derived from oxidative coupling of *p*-hydroxycinnamyl alcohols (monolignols) and related compounds. Lignification, that is, lignin deposition in apoplastic cell wall domains, is a pivotal biological function that early terrestrial plants acquired for land colonization. In particular, lignin is vital for the integrity of the thick secondary cell walls produced in xylem vascular tissues, such as tracheids, vessels, and fibers, where lignin is embedded with polysaccharides and confers essential mechanical properties to the cells required for water transport and structural support. Furthermore, plants execute lignification in other specialized cell types, such as seedcoats, the endothecium (lining of the anther lumen), endodermal cells, and secession cells for organ removal, and also in response to pathogen attack. Typically, 20–30% of plant dry matter, that is, lignocellulosic biomass, is made up of lignins. Hence, studies on lignin biosynthesis and bioengineering are motivated not only by its fundamental importance to plant development and physiology, but also by its agro-industrial importance in the context of plant biomass utilization [1].

Here we focus on the final step of lignin biosynthesis, in which monolignols secreted from cells are activated by cell-wall-localized oxidation systems, such as laccase/O_2 and peroxidase/H_2O_2. Lignin polymerization occurs via a combinatorial radical coupling process that is highly flexible in nature and allows plants to incorporate numerous lignin monomers—beyond the three classical monolignols, that is, coniferyl, sinapyl, and *p*-coumaryl alcohols—in different combinations to assemble different lignin polymers in different cell types. However, despite extensive research efforts, as recently reviewed in [2–4], many aspects of the mechanisms underlying the tight control of lignin polymerization *in planta* remain unclear. In this review, we provide a brief overview of the latest advances in our understanding of the biochemistry and cell biology aspects of lignin polymerization.

Lignin polymerization proceeds via simple combinatorial chemistry

Chemically, the key process of lignin polymerization involves so-called 'end-wise' polymerization in which oxidized monolignol radicals undergo cross-coupling reactions with radicals formed on the free-phenolic ends of growing lignin polymers. Permutations in the regiochemistry of the radical coupling step and variations in the mode of the post-coupling quinone methide rearomatization step allow numerous possible inter-monomeric linkages in the final lignin polymer (Figure 1a) [1]. *In vitro* lignin polymerization experiments that produce synthetic lignin polymers or dehydrogenation polymers (DHPs) via enzymatic or chemical oxidation of monolignols have demonstrated that the propensity for lignin linkage formation primarily depends on the chemical structure of monolignols [5–9]. Overall, the composition of different monolignols available in the polymerization site is likely the most important factor controlling the

Figure 1



(a) Monolignols and their polymerization. Lignin polymerization proceeds mainly via the 'end-wise' polymerization process in which an oxidized monolignol radical cross-couples with a radical formed on the free-phenolic ends of a growing lignin polymer. As an example, sequential polymer chain extension reactions via β–O–4 and β–5 radical coupling reactions with coniferyl and sinapy alcohols are illustrated. **(b)** Proteins comprising the lignin polymerization machinery. Laccase (LAC) and peroxidase (PRX) oxidative enzymes catalyze the phenoxy radical formations from monolignols. Apoplastic hydrogen peroxide, which is required in PRX-catalyzed oxidation, may be produced from apoplastic oxygen via two-step enzymatic reactions by NADPH oxidase, or Respiratory Burst Oxidase homolog (RBOH) protein, and superoxide dismutase (SOD). In the Casparian strip of Arabidopsis root endodermal cells, Casparian strip membrane domain proteins (CASPLs) are hypothesized to assemble the lignin polymerization proteins and guide where the Casparian strip forms. In addition, dirigent-domain-containing proteins (DIRs) are also implicated to function in directing the correct lignin depositions in the Casparian strips of Arabidopsis. **(c)** Localization of native-promoter-driven, mCherry-tagged LAC4 and PRX64 in cross sectional views of Arabidopsis interfascicular fibers. LAC4 and PRX64 display distinct localizations in the secondary cell wall and middle lamella cell wall domains, respectively, thus suggesting their functions are spatial separated during fiber lignification [56••].

structural organization of the final lignin polymer. This notion has been corroborated by theoretical studies using advanced computational methods [10–13] and generally supported by structural comparisons with various natural and genetically altered lignins incorporating different lignin monomer mixtures (citation for the COiB lignin review about 'Structure and Bioengineering'?). Additionally, numerous physio-chemical factors, such as monomer supply rate, pH, ionic strength, and concentration of polymerization enzymes and their capability to oxidize

different monomeric/polymeric substrates, influence the formation of DHPs [5,14,15,16•,17]. Notably, DHP formation can be affected by the presence of various cell-wall-associated polysaccharides [18–21]. Given that lignin polymerization *in planta* typically occurs after the deposition of polysaccharides in developing cell walls, pre-existing polysaccharide matrices may act as scaffolds for lignin polymerization and provide specific local environments within which monolignols undergo combinatorial radical coupling [20–22]. In fact, some polysaccharide-modified transgenic and mutant Arabidopsis lines display altered lignin deposition [23–25], while the impact of polysaccharide modifications on lignin chemical structures *in planta* is an active area of research.

Undeciphered role of dirigent proteins in lignification

The structural features of natural lignin polymers and their variability in mutant and transgenic plants may be addressed elsewhere in this issue of Current Opinion in Biotechnology (citation for the COiB lignin review about 'Structure and bioengineering'?). All the lignin structural data accumulated to date are in accordance with the view that the *in planta* lignin polymerization step occurs via simple combinatorial chemistry solely under chemical and physical control, that is, none of the proteins implicated in lignification exert a direct influence on the chemistry of lignin polymerization beyond the formation of phenoxy radicals [26]. The alternative hypothesis that a class of dirigent-domain-containing proteins (DIRs) control the primary structure of lignin [27], similarly to how DIRs control the regio/stereo-chemistries of analogous radical coupling reactions in the biosynthesis of lignans [28,29], still lacks sufficient biochemical and genetic evidence at this time. Nevertheless, some recent studies have implicated an intriguing function for DIRs in directing the spatial patterning of lignin deposition in specific cells [29–31]. In particular, the putative function of DIR10/ESB1 in the correct formation of the lignin-based Casparian strip in Arabidopsis roots is intriguing (Figure 1b) [30] (+citation for the COiB lignin review about 'Casparian strips'?). It is possible that, despite its inability to mediate the regio/stereo-selectivity of coniferyl alcohol coupling *in vitro* [32], DIR10/ESB1 may play a role in initiating lignin polymerization or localizing lignin pre-polymers in the Casparian strip, although whether DIR10/ESB1 can bind monolignols, lignin polymers, and/or their radicals is yet to be determined.

Respective roles of laccases and peroxidases in lignification

The combinatorial radical coupling of monolignols in cell walls may be initiated by the action of oxidative enzymes such as O_2-dependent laccases and H_2O_2-dependent peroxidases (Figure 1b). The catalytic promiscuity and large gene families of both enzyme types have long made it difficult to establish their functions *in planta* but recent studies have started to uncover the roles of specific laccases and peroxidases in lignin polymerization.

The essential role of laccases in the lignification of major xylem tissues in Arabidopsis was demonstrated through the characterization of multiple knockout mutants [3,33–35]. The *lac4 lac17* double mutants deficient in LACCASE4 (LAC4) and LAC17 functions showed hypolignified fibers and collapsed xylem vessel phenotypes in their inflorescence stems [33,34]. Additional loss of function of LAC11, in the *lac4 lac17 lac11* triple mutant, led to severe growth defects and failure to detect lignin in either stems or roots by histochemical analysis [35]. Overall, these laccase mutant studies have suggested that laccase activities are essential for lignin formation in the major xylem tissues of Arabidopsis and further analysis of this gene family and multiple mutant alleles will expand our understanding of laccase function in lignification. The tissue and cell type-specific expression patterns of laccase genes are post-transcriptionally regulated by microRNAs [36–38,39•]. For instance, overexpressing microRNA397 (miR397) in poplar [36] and Arabidopsis [37] and overexpressing miR528 in maize [39•] resulted in substantial reductions in lignin content along with reductions in transcript abundances of a subset of laccase genes. Recent bioengineering studies have highlighted laccase and laccase-specific microRNA genes as useful targets in biomass engineering for desirable lignin content and/or composition [39•,40–42].

Like laccases, peroxidases have been also suggested to have an active role in cell wall lignification in many plant species [3,43,44•]. In Arabidopsis, the lignification in the Casparian strip of root endodermal cells is largely mediated by PEROXIDASE64 (PRX64) because it is precisely localized to the Casparian strip and its microRNA-mediated cell-specific downregulation in root endodermis led to loss of Casparian strip function which depends on lignin deposition [45] (+citation for the COiB lignin review about 'Casparian strips'?). The same study also identified an NADPH oxidase or Respiratory Burst Oxidase homolog (RBOH) protein, RBOH-F, which produces the H_2O_2 required for PRX64-catalyzed lignin polymerization (Figure 1b). Furthermore, Casparian strip membrane domain proteins (CASPLs) are hypothesized to assemble other lignin polymerization proteins and thus guide where the Casparian strip forms (Figure 1b) [45]. Lignin accumulation in the floral abscission zone of Arabidopsis likely occurs via as yet unidentified peroxidases because mutations in RBOH-D/F or pharmacological inhibition of H_2O_2-dependent peroxidase function prevented lignin accumulation in secession cells developed in the abscission zone [46••].

Peroxidase function, however, is insufficient to rescue the severe hypolignification phenotypes observed in the xylem and fiber tissues of the *lac4 lac11 lac17* mutant

of Arabidopsis [35]. Nevertheless, single or double knockout mutants in several peroxidase genes exhibited typically minor but noticeable reductions in lignin content and/or altered lignin composition in Arabidopsis inflorescence stems [3,47–54]; notably, the *prx72* mutant showed ~35% lignin reduction and also sometimes displayed collapsed xylem vessel phenotypes with reduced stem heights [48]. These data suggest that, besides laccases, at least some of these peroxidases play a role in the lignification of xylem/fiber tissues in Arabidopsis.

A probable hypothesis for the respective functions of laccases and peroxidases during xylem lignification is that their functions are spatially and/or developmentally separated. Amongst many other laccases and peroxidases, *LAC4* and *PRX64* are the most highly expressed oxidative genes in Arabidopsis inflorescence stems along the lignification developmental gradient [55]. A recent subcellular localization analysis of native-promoter-driven, fluorescently-tagged LAC4 and PRX64 demonstrated that LAC4 is specifically localized throughout the secondary cell wall layers in fibers and vessels, whereas PRX64 localization is confined to the middle lamella and cell corners in fibers (Figure 1c) [56••]. These localization data suggest that LAC4 mediates lignin polymerization throughout the thick secondary cell wall layers whereas PRX64 functions primarily within the middle lamella in fibers. Considering the bulk of the biomass of Arabidopsis inflorescence stems is derived from secondary cell walls, this notion is in line with the dramatic loss of lignins in the *lac4 lac11 lac17* triple mutant in contrast to the limited lignin depletion in the peroxidase mutants analyzed to date [35]. However, no *prx64* mutants have yet been described and thus the function of PRX64 in the lignification of xylem/fiber tissues in Arabidopsis remains unclear. It is also noteworthy that ZPO-C, a Zinnia PRX64/66/72 homolog peroxidase, was shown to be localized to the secondary cell walls of xylem vessels and was also specifically expressed in developing xylem vessels in Zinnia [57]. This contrasts the fiber-specific expression of PRX64 in Arabidopsis, although both proteins appeared to be localized to primary cell wall domains in tricellular junctions [56••,57]. Overall, future studies should further closely investigate the spatio-temporal localization of the diverse laccase and peroxidase proteins implicated in lignification.

Localization of the polymerization machinery directs the spatial control of lignification

The mechanisms controlling the spatio-temporal patterning of lignin polymerization remain largely elusive. During protoxylem tracheary element (TE) formation, for example, lignin is precisely deposited in the spiral secondary cell walls while the intervening primary cell walls remain lignin free. In Arabidopsis, laccase genes, such as *LAC4*, *LAC11*, and *LAC17*, are specifically expressed during the onset of secondary cell wall formation [55] and LAC4 and LAC17 have been shown to be precisely localized to secondary cell wall domains during protoxylem TE formation [58]. Furthermore, LAC4 is highly immobile post secretion to secondary cell walls as recently determined by a fluorescence recovery after photobleaching (FRAP) analysis [56••], suggesting that LAC4 is specifically secreted and tightly anchored to polysaccharide-rich secondary cell wall domains [56••,58]. In contrast, recent imaging analyses of lignin polymerization *in planta* using chemical probes, such as fluorescently-tagged [58,59] and chemical-reporter-tagged monolignols [60,61,62•], have suggested that monolignols are highly mobile once exported to the apoplast.

Collectively, the precise localization of oxidative enzymes in specific cell wall domains is likely the key determinant of the spatial patterning of lignin deposition (Figure 1c) [56••,58]. This model is in good agreement with the model proposed for lignin deposition in the Casparian strip in root endodermis cells, where CASPLs position the lignin polymerization machinery, including PRX64, RBOH-F and DIR10/EFB1, by forming a protein scaffold to capture and polymerize monolignols at the site where the Casparian strip forms [45] (+citation for the COiB lignin review about 'Casparian strips'?). In turn, the spatial variability in the deposition of different lignin polymers derived from different monolignol precursor mixtures [2–4], is not necessarily directed by the monomer specificity of the polymerization machinery but is also (more likely) controlled by the local supply of monolignols, that is, by the controlled expression of genes responsible for monolignol synthesis and/or transportation, in lignifying cells and/or in neighboring cells that supply monolignols (citation for the COiB lignin review about 'Transportation'?).

Conclusions

Although the actual polymerization process of monolignols proceeds via simple combinatorial chemistry, the processes in which different monolignols are assembled and activated for lignin polymerization are under tight biological control. The emerging view is that specific laccase and/or peroxidase proteins are secreted to facilitate the spatial control of lignin deposition in distinct cell wall domains. However, to what degree their substrate specificities influence the incorporation of different monolignol types into lignin polymers remains unknown; although several *in vitro* peroxidase/laccase catalytic assay and DHP preparation studies reported to date used recombinant enzymes expressed in bacterial systems (e.g., with *Escherichia coli*), it is also important to test their functions by using enzymes expressed with eukaryotic systems that facilitate the *N*-glycosylations essential for these extracellular glycoproteins [63,64]. Besides, it is still to be determined how other possible protein members comprising the polymerization machinery (e.g., RBOHs,

superoxide dismutase, DIRs and/or CASPLs), reactive oxygen species, polysaccharide matrices and any other molecules present in the lignin polymerization site contribute to the assembly and polymerization of monolignols in different cell types. There is a need for further high-resolution analysis of gene expression in specific cell types as well as of protein localization and lignin chemical structure down to individual cell wall layers. By developing a unified view of how plants elaborately execute this last act in cell wall lignification, we may be able to more precisely manipulate cell wall structures for sustainable production of biofuels and biomass-based materials.

Conflict of interest statement

Nothing declared.

Acknowledgements

The authors apologize to all colleagues whose important contributions could not be highlighted or discussed herein due to space limitations. We thank Drs. John Ralph, Wout Boerjan, Lacey Samuels, Toshiaki Umezawa, and Hideto Hiraide for helpful discussions. We also thank the two anonymous reviewers for providing insightful comments. Related work in the laboratory of YT is supported by the Japan Society for the Promotion of Science (KAKENHI, #16H06198 and #16K14958) and KURCA (Future Development Funding Program FY2018) and RISH (the Mission-linked Research Funding, #2016-5-2-1), Kyoto University.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Mottiar Y, Vanholme R, Boerjan W, Ralph J, Mansfield SD: **Designer lignins: harnessing the plasticity of lignification**. *Curr Opin Biotechnol* 2016, **37**:190-200.

2. Schuetz M, Smith R, Ellis B: **Xylem tissue specification, patterning, and differentiation mechanisms**. *J Exp Bot* 2013, **64**:11-31.

3. Barros J, Serk H, Granlund I, Pesquet E: **The cell biology of lignification in higher plants**. *Ann Bot* 2015, **115**:1053-1074.

4. Meents MJ, Watanabe Y, Samuels AL: **The cell biology of secondary cell wall biosynthesis**. *Ann Bot* 2018, **121**:1107-1125.

5. Demont-Caulet N, Lapierre C, Jouanin L, Baumberger S, Mechin V: **Arabidopsis peroxidase-catalyzed copolymerization of coniferyl and sinapyl alcohols: kinetics of an endwise process**. *Phytochemistry* 2010, **71**:1673-1683.

6. Kishimoto T, Chiba W, Saito K, Fukushima K, Uraki Y, Ubukata M: **Influence of syringyl to guaiacyl ratio on the structure of natural and synthetic lignins**. *J Agric Food Chem* 2010, **58**:895-901.

7. Tobimatsu Y, Chen F, Nakashima J, Jackson L, Escamilla-Treviño LL, Dixon RA, Ralph J: **Coexistence but independent biosynthesis of catechyl and guaiacyl/syringyl lignins in plant seeds**. *Plant Cell* 2013, **25**:2587-2600.

8. Kishimoto T, Takahashi N, Hamada M, Nakajima N: **Biomimetic oxidative coupling of sinapyl acetate by silver oxide: preferential formation of β-O-4 type structures**. *J Agric Food Chem* 2015, **63**:2277-2283.

9. Harman-Ware AE, Happs RM, Davison BH, Davis MF: **The effect of coumaryl alcohol incorporation on the structure and composition of lignin dehydrogenation polymers**. *Biotechnol Biofuels* 2017, **10**.

10. van Parijs FRD, Morreel K, Ralph J, Boerjan W, Merks RMH: **Modeling lignin polymerization. I. Simulation model of dehydrogenation polymers**. *Plant Physiol* 2010, **153**:1332-1344.

11. Sangha AK, Parks JM, Standaert RF, Ziebell A, Davis M, Smith JC: **Radical coupling reactions in lignin synthesis: a density functional theory study**. *J Phys Chem B* 2012, **116**:4760-4768.

12. Sangha AK, Davison BH, Standaert RF, Davis MF, Smith JC, Parks JM: **Chemical factors that control lignin polymerization**. *J Phys Chem B* 2014, **118**:164-170.

13. Berstis L, Elder T, Crowley M, Beckham GT: **Radical nature of C-lignin**. *ACS Sustain Chem Eng* 2016, **4**:5327-5335.

14. Tobimatsu Y, Takano T, Kamitakahara H, Nakatsubo F: **Reactivity of syringyl quinone methide intermediates in dehydrogenative polymerization. Part 2: pH effect in horseradish peroxidase-catalyzed polymerization of sinapyl alcohol**. *Holzforschung* 2010, **64**:183-192.

15. Hwang H, Moon SJ, Won K, Kim YH, Choi JW: **Parameters affecting in vitro monolignol couplings during dehydrogenative polymerization in the presence of peroxidase and H_2O_2**. *J Ind Eng Chem* 2015, **26**:390-395.

16. • Warinowski T, Koutaniemi S, Karkonen A, Sundberg I, Toikka M, Simola LK, Kilpelainen I, Teeri TH: **Peroxidases bound to the growing lignin polymer produce natural like extracellular lignin in a cell culture of Norway spruce**. *Front Plant Sci* 2016, **7**:1523.
A lignifying norway spruce cell culture system was used to isolate oxidative enzymes that were then used to make synthetic lignins for subsequent structural analysis. The data suggested that a group of apoplastic, polymer-oxidizing peroxidases bind to the lignin polymer and are responsible for production of natural-like lignin in the cell culture system.

17. Shigeto J, Honjo H, Fujita K, Tsutsumi Y: **Generation of lignin polymer models via dehydrogenative polymerization of coniferyl alcohol and syringyl alcohol via several plant peroxidases involved in lignification and analysis of the resulting DHPs by MALDI-TOF analysis**. *Holzforschung* 2018, **72**:267-274.

18. Terashima N, Atalla RH, Ralph SA, Landucci LL, Lapierre C, Monties B: **New preparations of lignin polymer models under conditions that approximate cell-wall lignification. 1. Synthesis of novel lignin polymer models and their structural characterization by ^{13}C NMR**. *Holzforschung* 1995, **49**:521-527.

19. Cathala B, Monties B: **Influence of pectins on the solubility and the molar mass distribution of dehydrogenative polymers (DHPs, lignin model compounds)**. *Int J Biol Macromol* 2001, **29**:45-51.

20. Li Q, Koda K, Yoshinaga A, Takabe K, Shimomura M, Hirai Y, Tamai Y, Uraki Y: **Dehydrogenative polymerization of coniferyl alcohol in artificial polysaccharides matrices: effects of xylan on the polymerization**. *J Agric Food Chem* 2015, **63**:4613-4620.

21. Aminzadeh S, Zhang LM, Henriksson G: **A possible explanation for the structural inhomogeneity of lignin in LCC networks**. *Wood Sci Technol* 2017, **51**:1365-1376.

22. Donaldson LA, Knox JP: **Localization of cell wall polysaccharides in normal and compression wood of radiata pine: relationships with lignification and microfibril orientation**. *Plant Physiol* 2012, **158**:642-653.

23. Petersen PD, Lau J, Ebert B, Yang F, Verhertbruggen Y, Kim JS, Varanasi P, Suttangkakul A, Auer M, Loque D *et al.*: **Engineering of plants with improved properties as biofuels feedstocks by vessel-specific complementation of xylan biosynthesis mutants**. *Biotechnol Biofuels* 2012, **5**.

24. Hao ZY, Avci U, Tan L, Zhu X, Glushka J, Pattathil S, Eberhard SF, Sholes T, Rothstein GE, Lukowitz WL *et al.*: **Loss of Arabidopsis GAUT12/IRX8 causes anther indehiscence and leads to reduced G lignin associated with altered matrix polysaccharide deposition**. *Front Plant Sci* 2014, **5**.

25. Voxeur A, Soubigou-Taconnat L, Legee F, Sakai K, Antelme S, Durand-Tardif M, Lapierre C, Sibout R: **Altered lignification in *mur1-1* a mutant deficient in GDP-L-fucose synthesis with reduced RG-II cross linking**. *Plos One* 2017, **12**.

26. Ralph J, Brunow G, Harris PJ, Dixon RA, Schatz PF, Boerjan W: **Lignification: are lignins biosynthesized via simple combinatorial chemistry or via proteinaceous control and template replication?** In *Recent Advances in Polyphenol Research*, Vol. 1. Edited by Daayf F, El Hadrami A, Adam L, Ballance GM. Wiley-Blackwell Publishing; 2008:36-66.

27. Davin LB, Jourdes M, Patten AM, Kim KW, Vassao DG, Lewis NG: **Dissection of lignin macromolecular configuration and assembly: comparison to related biochemical processes in allyl/propenyl phenol and lignan biosynthesis**. *Nat Prod Rep* 2008, **25**:1015-1090.

28. Davin LB, Wang HB, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG: **Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center**. *Science* 1997, **275**:362-366.

29. Paniagua C, Bilkova A, Jackson P, Dabravolski S, Riber W, Didi V, Houser J, Gigli-Bisceglia N, Wimmerova M, Budinska E *et al.*: **Dirigent proteins in plants: modulating cell wall metabolism during abiotic and biotic stress exposure**. *J Exp Bot* 2017, **68**:3287-3301.

30. Hosmani PS, Kamiya T, Danku J, Naseer S, Geldner N, Guerinot ML, Salt DE: **Dirigent domain-containing protein is part of the machinery required for formation of the lignin-based casparian strip in the root**. *Proc Nat Acad Sci U S A* 2013, **110**:14498-14503.

31. Funatsuki H, Suzuki M, Hirose A, Inaba H, Yamada T, Hajika M, Komatsu K, Katayama T, Sayama T, Ishimoto M *et al.*: **Molecular basis of a shattering resistance boosting global dissemination of soybean**. *Proc Nat Acad Sci U S A* 2014, **111**:17797-17802.

32. Kim KW, Moinuddin SGA, Atwell KM, Costa MA, Davin LB, Lewis NG: **Opposite stereoselectivities of dirigent proteins in Arabidopsis and Schizandra species**. *J Biol Chem* 2012, **287**:33957-33972.

33. Berthet S, Demont-Caulet N, Pollet B, Bidzinski P, Cezard L, Le Bris P, Borrega N, Herve J, Blondet E, Balzergue S *et al.*: **Disruption of LACCASE4 and 17 results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems**. *Plant Cell* 2011, **23**:1124-1137.

34. Berthet S, Thevenin J, Baratiny D, Demont-Caulet N, Debeaujon I, Bidzinski P, Leple JC, Huis R, Hawkins S, Gomez LD *et al.*: **Role of plant laccases in lignin polymerization**. *Adv Bot Res* 2012, **61**:145-172.

35. Zhao Q, Nakashima J, Chen F, Yin YB, Fu CX, Yun JF, Shao H, Wang XQ, Wang ZY, Dixon RA: **LACCASE is necessary and nonredundant with PEROXIDASE for lignin polymerization during vascular development in Arabidopsis**. *Plant Cell* 2013, **25**:3976-3987.

36. Lu SF, Li QZ, Wei HR, Chang MJ, Tunlaya-Anukit S, Kim H, Liu J, Song JY, Sun YH, Yuan LC *et al.*: **Ptr-miR397a is a negative regulator of laccase genes affecting lignin content in *Populus trichocarpa***. *Proc Nat Acad Sci U S A* 2013, **110**:10848-10853.

37. Wang CY, Zhang SC, Yu Y, Luo YC, Liu Q, Ju CL, Zhang YC, Qu LH, Lucas WJ, Wang XJ *et al.*: **MiR397b regulates both lignin content and seed number in Arabidopsis via modulating a laccase involved in lignin biosynthesis**. *Plant Biotechnol J* 2014, **12**:1132-1142.

38. Zhao YY, Lin S, Qiu ZB, Cao DC, Wen JL, Deng X, Wang XH, Lin JX, Li XJ: **MicroRNA857 is involved in the regulation of secondary growth of vascular tissues in Arabidopsis**. *Plant Physiol* 2015, **169**:2539-2552.

39. • Sun Q, Liu XG, Yang J, Liu WW, Du QG, Wang HQ, Fu CX, Li WX: **MicroRNA528 affects lodging resistance of maize by regulating lignin biosynthesis under nitrogen-luxury conditions**. *Mol Plant* 2018, **11**:806-814.

Mechanistic insight was provided into maize lodging that is regulated by a monocot-specific microRNA which targets LACCASE genes required for lignification of vascular tissues. Knockdown of*ZmmiR528* significantly increased the lignin content and rind penetrometer resistance of maize stems, whereas transgenic maize lines overexpressing *ZmmiR528* had reduced lignin content and rind penetrometer resistance and were thus prone to lodging.

40. Wang JH, Feng JJ, Jia WT, Chang S, Li SZ, Li YX: **Lignin engineering through laccase modification: a promising field for energy plant improvement**. *Biotechnol Biofuels* 2015, **8**:145.

41. Bryan AC, Jawdy S, Gunter L, Gjersing E, Sykes R, Hinchee MAW, Winkeler KA, Collins CM, Engle N, Tschaplinski TJ *et al.*: **Knockdown of a laccase in *Populus deltoides* confers altered cell wall chemistry and increased sugar release**. *Plant Biotechnol J* 2016, **14**:2010-2020.

42. Wang JH, Feng JJ, Jia WT, Fan PX, Bao H, Li SZ, Li YX: **Genome-wide identification of *Sorghum bicolor* laccases reveals potential targets for lignin modification**. *Front Plant Sci* 2017, **8**.

43. Shigeto J, Tsutsumi Y: **Diverse functions and reactions of class III peroxidases**. *New Phytol* 2016, **209**:1395-1402.

44. • Laitinen T, Morreel K, Delhomme N, Gauthier A, Schiffthaler B, Nickolov K, Brader G, Lim KJ, Teeri TH, Street NR *et al.*: **A key role for apoplastic H_2O_2 in Norway spruce phenolic metabolism**. *Plant Physiol* 2017, **174**:1449-1475.

This study utilized an extracellular-lignin-forming cell culture of Norway spruce and chemical inhibitors coupled to metabolite and transcriptomic analyses in order to dissect the role of apoplastic H_2O_2 in gymnosperm phenolic metabolism. In line with the notion that peroxidases activate monolignols for lignin polymerization in this plant system, scavenging of apoplastic H_2O_2 repressed extracellular-lignin formation, along with remodulation of the transcriptome with reduced carbon flux into the monolignol biosynthetic pathway.

45. Lee Y, Rubio MC, Alassimone J, Geldner N: **A mechanism for localized lignin deposition in the endodermis**. *Cell* 2013, **153**:402-412.

46. •• Lee Y, Yoon TH, Lee J, Jeon SY, Lee JH, Lee MK, Chen HZ, Yun J, Oh SY, Wen XH *et al.*: **A lignin molecular brace controls precision processing of cell walls critical for surface integrity in Arabidopsis**. *Cell* 2018, **173**:1468-1480.

The coordinated lignification of secession cells in the floral abscission zone of Arabidopsis provides a honeycomb structure of lignin which acts as a mechanical 'brace' to localize cell wall breakdown and correctly guides cell-cell detachment. Loss of the lignin brace via loss-of-function of RBOH NADPH oxidases leads to inproper floral separation, surface barrier defects and susceptibility to pathogen infection.

47. Herrero J, Esteban-Carrasco A, Zapata JM: **Looking for *Arabidopsis thaliana* peroxidases involved in lignin biosynthesis**. *Plant Physiol Biochem* 2013, **67**:77-86.

48. Herrero J, Fernández-Pérez F, Yebra T, Novo-Uzal E, Pomar F, Pedreño MA, Cuello J, Guéra A, Esteban-Carrasco A, Zapata JM: **Bioinformatic and functional characterization of the basic peroxidase 72 from Arabidopsis thaliana involved in lignin biosynthesis**. *Planta* 2013, **237**:1599-1612.

49. Shigeto J, Kiyonaga Y, Fujita K, Kondo R, Tsutsumi Y: **Putative cationic cell-wall-bound peroxidase homologues in Arabidopsis, AtPrx2, AtPrx25, and AtPrx71, are involved in lignification**. *J Agric Food Chem* 2013, **61**:3781-3788.

50. Fernandez-Perez F, Pomar F, Pedreno MA, Novo-Uzal E: **Suppression of Arabidopsis peroxidase 72 alters cell wall and phenylpropanoid metabolism**. *Plant Sci* 2015, **239**:192-199.

51. Fernandez-Perez F, Pomar F, Pedreno MA, Novo-Uzal E: **The suppression of AtPrx52 affects fibers but not xylem lignification in Arabidopsis by altering the proportion of syringyl units**. *Physiol Plant* 2015, **154**:395-406.

52. Fernandez-Perez F, Vivar T, Pomar F, Pedreno MA, Novo-Uzal E: **Peroxidase 4 is involved in syringyl lignin formation in *Arabidopsis thaliana***. *J Plant Physiol* 2015, **175**:86-94.

53. Shigeto J, Itoh Y, Hirao S, Ohira K, Fujita K, Tsutsumi Y: **Simultaneously disrupting AtPrx2, AtPrx25 and AtPrx71 alters lignin content and structure in Arabidopsis stem**. *J Int Plant Biol* 2015, **57**:349-356.

54. Cosio C, Ranocha P, Francoz E, Burlat V, Zheng Y, Perry SE, Ripoll JJ, Yanofsky M, Dunand C: **The class III peroxidase PRX17 is a direct target of the MADS-box transcription factor AGAMOUS-LIKE15 (AGL15) and participates in lignified tissue formation**. *New Phytol* 2017, **213**:250-263.

55. Hall H, Ellis B: **Transcriptional programming during cell wall maturation in the expanding Arabidopsis stem**. *BMC Plant Biol* 2013, **13**.

56. •• Chou EY, Schuetz M, Hoffmann N, Watanabe Y, Sibout R, Samuels AL: **Distribution, mobility, and anchoring of lignin-related oxidative enzymes in Arabidopsis secondary cell walls**. *J Exp Bot* 2018, **69**:1849-1859.
This study documents the subcellular localization of LACCASE4 and PEROXIDASE64 in Arabidopsis inflorescence stems. LAC4 and PRX64 have complementary localization in the secondary cell wall and middle lamella cell wall domains in Arabidopsis fiber cells, respectively. FRAP analysis determined that LAC4 is tightly anchored to the polysaccharide rich secondary cell wall domains and thus cannot be remobilized once secreted.

57. Sato Y, Demura T, Yamawaki K, Inoue Y, Sato S, Sugiyama M, Fukuda H: **Isolation and characterization of a novel peroxidase gene *ZPO-C* whose expression and function are closely associated with lignification during tracheary element differentiation**. *Plant Cell Physiol* 2006, **47**:493-503.

58. Schuetz M, Benske A, Smith RA, Watanabe Y, Tobimatsu Y, Ralph J, Demura T, Ellis B, Samuels AL: **Laccases direct lignification in the discrete secondary cell wall domains of protoxylem**. *Plant Physiol* 2014, **166** 798-U489.

59. Tobimatsu Y, Wagner A, Donaldson L, Mitra P, Niculaes C, Dima O, Kim JI, Anderson N, Loque D, Boerjan W *et al.*: **Visualization of plant cell wall lignification using fluorescence-tagged monolignols**. *Plant J* 2013, **76**:357-366.

60. Tobimatsu Y, Van de Wouwer D, Allen E, Kumpf R, Vanholme B, Boerjan W, Ralph J: **A click chemistry strategy for visualization of plant cell wall lignification**. *Chem Commun* 2014, **50**:12262-12265.

61. Pandey JL, Kiemle SN, Richard TL, Zhu YM, Cosgrove DJ, Anderson CT: **Investigating biochemical and developmental dependencies of lignification with a click-compatible monolignol analog in *Arabidopsis thaliana* stems**. *Front Plant Sci* 2016, **7**.

62. • Lion C, Simon C, Huss B, Blervacq AS, Tirot L, Toybou D, Spriet C, Slomianny C, Guerardel Y, Hawkins S *et al.*: **BLISS: a bioorthogonal dual-labeling strategy to unravel lignification dynamics in plants**. *Cell Chem Biol* 2017, **24**:326-338.
The authors reported a multiplexed, chemical labeling method to visualize plant cell wall lignification*in planta* by using two different monolignol mimics tagged with azide and alkyne reporters that can be independently incorporated into cell walls, then differentially derivatized *in vivo* via two bioorthogonal click reactions. As a proof of concept, they examined the incorporation of H- and G-type monolignol mimics into flax lignifying tissues.

63. Turlapati PV, Kim K-W, Davin LB, Lewis NG: **The laccase multigene family in *Arabidopsis thaliana*: towards addressing the mystery of their gene function(s)**. *Planta* 2011, **233**:439-470.

64. Welinder KG, Justesen AF, Kjærsgård IVH, Jensen RB, Rasmussen SK, Jespersen HM, Duroux L: **Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana***. *Euro J Biochem* 2002, **269**:6063-6081.